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Diversification of Circum-Mediterranean Barbels

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1. Introduction

The Mediterranean Basin is one of the 25 most biodiverse regions on Earth. It is considered a biodiversity hotspot for its high numbers of endemic vascular plants, birds, mammals, reptiles, and amphibians, sometimes restricted to small distribution areas (Médail & Quézel, 1999; Mittermeier et al., 1998; Myers et al., 2000). The Mediterranean has had a long and complex geomorphologic history, being a relic of the Mesozoic Tethys Ocean. The Tethys had disappeared by the end of the Eocene (34 Ma) due to the collision of the Indian and Asian plates (Rögl, 1999). The orogenic movements raised new mountain ranges in the Taurides, the Hellenides, the Dinarides and finally the Alps by the Middle/early Late Miocene (Hsü et al., 1977). This orogeny separated the borning Mediterranean and a central/eastern European inland sea – the Paratethys Sea (Hsü et al., 1977; Rögl, 1999). Landbridge connections and seaway passages between the Mediterranean and Paratethys, and between them and the Indian and Atlantic oceans, were then intermittent throughout the Miocene until the final opening of the Strait of Gibraltar ending the Messinian Salinity Crisis (Agustí et al., 2006; Hsü et al., 1977; Krijgsman et al., 1999; Rögl, 1999). This complex geomorphological scenario has allowed multiple faunal and floral exchanges between neighboring regions (e.g. Agustí et al., 2006; Benammi et al., 1996; Pickford et al., 1993, 1995); this melting pot might have contributed to the extraordinary diversity observed nowadays. For instance, the Middle East has been an important region for freshwater fish interchange between Africa, Asia and Europe (Durand et al., 2002). Another relevant aspect is whether persistence (i.e. low extinction), diversification (i.e. high speciation), or both, are responsible for high species diversity in the Mediterranean (e.g. Reyjol et al., 2007).

Among vertebrate groups, primary freshwater fishes probably constitute the majority of living endemisms in the Mediterranean region and include several species with restricted distribution ranges. Certain regions in the northern Mediterranean have been identified as important biodiversity hotspots for riverine fish (Reyjol et al., 2007) and the same is likely true for southern Mediterranean ones. This is explained by the limited dispersal routes of freshwater-restricted species, living within the confines imposed by salt water on one hand, and land on the other. Such qualities make primary freshwater fishes ideal models for the study of biogeographical history, landscape evolution and processes driving diversification in general (Briggs, 1995). Cyprinid fishes are a prime example. They are the most diverse family within the order Cypriniformes and naturally inhabit freshwaters of all continents except for Antarctica, Australia, and South America (Banarescu & Coad,

1991). Therefore, they can offer invaluable insights on the historical biogeography of the Mediterranean Basin.

Two major biogeographical scenarios have been proposed to explain current distribution of Mediterranean cyprinids. The classical northern river dispersal hypothesis states that primary freshwater fishes reached Europe from Asia when the Turgai Sea dried out, and then continuously dispersed southwards from Europe to Africa by river rearrangements, throughout the Miocene and Pliocene (Almaça, 1976, 1988; Banarescu, 1960, 1992). On the other hand, the southern sea hypothesis proposes that cyprinids colonized different regions across the Mediterranean. Some proponents of the latter favor a dispersalist scenario during the Messinian Salinity Crisis (Bianco, 1990), others a vicariant one from the Middle East, to Africa, to Iberia through intercontinental land bridges during the formation of the current North African coast in the early Pliocene (Doadrio, 1990), or a combination of both dispersal and vicariance (Zardoya & Doadrio, 1999).

In the present work, the timing and pattern of diversification of circum-Mediterranean barbels is re-examined using molecular phylogenies and the latest fossil data available to shed light on historical biogeography of the region and how it shaped the evolution of freshwater fishes.

2. Barbels as a model system in Mediterranean biogeography studies

After decades during which the genus *Barbus* was used for many different barbines, from Asia, Africa or Europe alike, *Barbus s. str.* was restricted to 'true' barbels. 'True' barbels are a group of tetraploid fishes distributed throughout freshwaters of Europe, southwestern Asia and northern Africa (Berrebi et al., 1996; Collares-Pereira & Madeira, 1990; Howes, 1987; Ráb & Collares-Pereira, 1995). They are composed of two lineages based on morphology (Doadrio, 1990) and molecular evidence (e.g. Gante, 2009; Machordom & Doadrio, 2001a). These lineages, *Barbus* and *Luciobarbus*, are now considered separate genera by several authors, nomenclature that is followed here. Based on mitochondrial phylogenies, Tsigenopoulos & Berrebi (2000) showed that the monotypic tetraploid genus *Aulopyge* is sister to *Barbus* and *Luciobarbus*. Soon after, Durand et al. (2002) and Tsigenopoulos et al. (2003) found that the hexaploid genus *Capoeta* nests within *Luciobarbus*. Thus, the delimitation of 'barbel' is loosened to include the Balkanic *Aulopyge huegelii* and the Middle Eastern *Capoeta* in the present analysis.

Barbels are medium- to large-sized bottom dweller fishes, adapted to a variety of habitats from standing water lakes to fast-flowing montane rivers. Taken together, these four genera have a very wide geographical range, from the Black Sea, Caucasus and Middle East to the Iberian Peninsula in the West, from northwestern Africa in the South to Russia in the North. In the Mediterranean region they are only not found in Libya and Egypt. Interestingly, the four genera are mostly allopatric except for the Caucasus, Middle East, and small areas in Anatolia, Greece, and Iberia. In particular, *Luciobarbus* is found in the Caucasus, Middle East, Anatolia, Greece, northwestern Africa and Iberia. This truly circum-Mediterranean distribution is ideal for the study of historical biogeography of the region, since it allows tracing time and direction of colonization events between different regions. Also, barbels as a whole are a diverse group with dozens of species with distributions usually restricted to specific basins. This offers the opportunity for repeated sampling from each region. Finally, a sizeable collection of mitochondrial sequences of barbels and allied genera has been deposited in public repositories by many authors over the years. They are an invaluable resource.

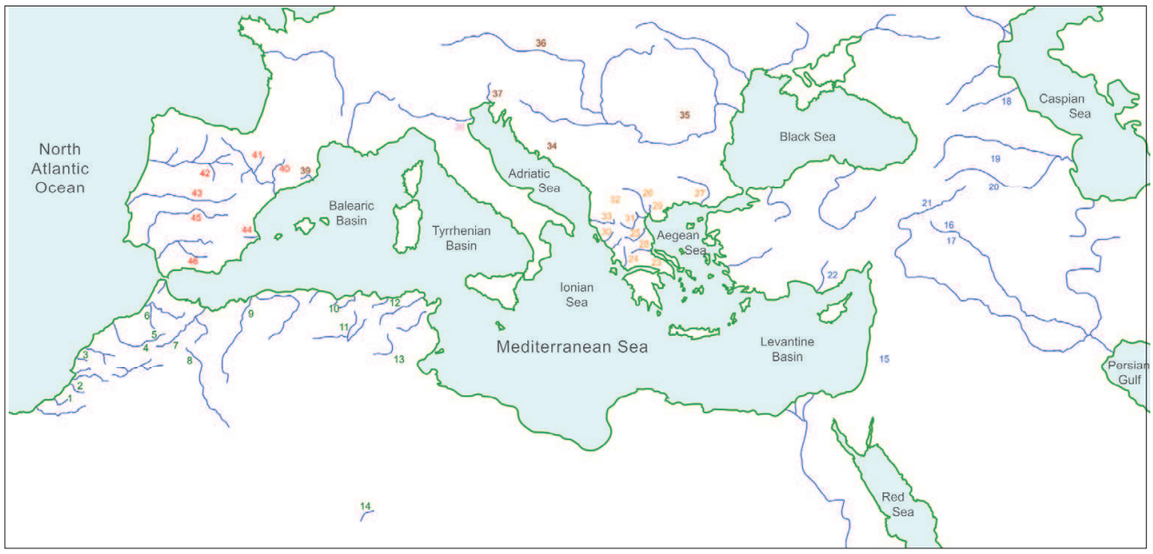

Fig. 1. Sampling sites of *Barbus*, *Luciobarbus*, *Capoeta* and *Aulopyge huegelii* analyzed in the present study. Numbers refer to Table 1. Color codes refer to geographical range of the species irrespective of generic placement: Northern Africa, Anatolia, Middle East and Caucasus, Greece, central and eastern Europe, and Balkans, Italy, and Iberia.

3. Improved fossil data and calibration of barbel phylogenies

Some previous studies of historical Mediterranean biogeography – of parts or the whole area – have made use of calibrated molecular phylogenies of barbels. Most of the studies relied on molecular clocks calibrated with known geological events, such as the openings of the Strait of Gibraltar and/or the Strait of Korinthos (Machordom & Doadrio, 2001b; Mesquita et al., 2007; Tsigenopoulos et al., 2003, 2010; Zardoya & Doadrio, 1999). The results from the different studies varied slightly, depending on which particular node was calibrated, but most importantly they might have inadvertently biased results for accepted vicariant events or those perceived as more likely.

Other studies have calibrated molecular phylogenies using published rates for other organisms (Durand et al., 2002; Tsigenopoulos & Berrebi, 2000; Tsigenopoulos et al. 2010). The use of (a range of) possible mutation rates is commonly accepted, in particular if fossils are not available for specific groups. Nevertheless, mass-specific metabolic rate and temperature influence the rate of molecular evolution in poikilotherm fishes (Estabrook et al., 2007). Using a 'universal' rate might have as a consequence over- or underestimation of the real rate of molecular evolution of the particular study organism. Interestingly, of the abovementioned studies that calibrated phylogenies either using known geological events or published rates, perhaps all but one underestimated the age of *Barbus* and *Luciobarbus* according to current fossil data.

One last study calibrated a molecular phylogeny with fossil data (Gante et al., 2009), but it was restricted to a very small area of the Mediterranean Basin. Therefore, given the current scenario just described, there is the need for a new analysis using updated fossil information, across the entire Mediterranean Basin, to re-evaluate the timing and pattern of diversification of circum-Mediterranean barbels.

Latest fossil data available in Böhme & Ilg (2003) were used to calibrate a molecular phylogeny. Divergence times and their credibility intervals (highest posterior density: HPD)

were estimated using a bayesian MCMC approach implemented in BEAST v1.6.1 (Drummond & Rambaut, 2007). *Barbus* fossils of Burdigalian age are now known from several localities in what is presently Central Europe and Anatolia (oldest: 19.0 Ma). This wide distribution area suggests that the genus had already diversified by the Early Miocene. Likewise, *Luciobarbus* fossils of Burdigalian age are known from Anatolia (oldest: 17.7 Ma). These dates set hard lower bounds for the diversification of each group. Additionally, *Luciobarbus* fossils of Messinian age are known from the Iberian Peninsula (oldest: 5.8 Ma), which represents a lower bound for the diversification of Iberian *Luciobarbus* as in Gante et al. (2009). The upper age is a soft bound free to vary following a lognormal distribution (Ho, 2007) set in real space with average of 1.0 and standard deviation of 0.5. Each gene used (see below) was a distinct data partition, with unlinked substitution models, and following relaxed uncorrelated lognormal clock models and a General Time Reversible model of evolution. Third codon positions were treated separately from 1st and 2nd codon positions. A speciation birth-death tree prior was used, since a Yule speciation prior assumes complete taxon sampling. Analysis was run for 25,000,000 generations, sampled every 2,500 generations, first 1,001 trees discarded as burn-in.

A total of 80 taxa were analyzed for the mitochondrial regions cytochrome *b* (1,141 bp) and ATPsynthases 6/8 (842 bp). As target ingroup, for the reasons explained above, representatives of *Barbus* (n=16), *Luciobarbus* (n=29), *Capoeta* (n=3), and *Aulopyge huegelii* from throughout the distribution area of the group were included. Additional cyprinins (n=31) originating in Asia and Africa were included in the analysis to provide a geographic, as well as phylogenetic context. Since the birth-death tree prior used assumes balanced sampling, outgroup species with varying divergence levels were selected (Fig. 1; Table 1).

Species	Fig. 1	River	Locality/Region	ATP synthase6/8	Cytochrome *b*	Source
Aulopyge huegelii	34	Busko Lake	Bosnia-Herzegovina	AF287359	AF287415	a
Barbus balcanicus	37	Judrio	Gorizia (Italy)	AF287368	AF287424	b
Barbus cf. *balcanicus*	31	Aliakmon	Kaloneri (Greece)	AF287392	AF287439	a
Barbus barbus	36	Danube	Lutzmannsburg (Austria)	AB238965	AB238965	c
Barbus carpathicus	35	Dîmbovitza	Dragomiresti (Romania)	AF287397	AF287441	a
Barbus cyclolepis	27	Erithropotamus	Mikro Derio (Greece)	AF287372	AF090782	a,d
Barbus euboicus	28	Maniklotiko	Oxilothos (Greece)	AF287378	AF090785	a,d
Barbus haasi	40	Esca	Isaba (Spain)	AY004687	AF045976	b,e
Barbus macedonicus	26	Axios	Axiopolis (Greece)	AY004720	AY004753	b
Barbus meridionalis	39	Tordera	Barcelona (Spain)	AF287386	AF045977	a,e
Barbus peloponnesius	30	Thiamis	Parapotamus (Greece)	AF287390	AF287438	a
Barbus plebejus	38	Roggia	Udine (Italy)	AY004717	AY004750	a
Barbus prespensis	32	Prespa Lake	Psarades (Greece)	AF287400	AF090790	a,d
Barbus rebeli	33	Aoos	Komitsa (Greece)	AF287401	AF090791	a,d
Barbus sperchiensis	28	Sperchios	Lamia (Greece)	AF287374	AF090783	a,d
Barbus strumicae	26	Agiaki	Kastanies (Greece)	AF287375	AF090784	a,d
Barbus thessalus	25	Pinios	Omolio (Greece)	AF287365	AF090781	a,d
Capoeta angorae	22	Seyhan	Turkey	–	AF145950	f
Capoeta capoeta	19	Sevan Lake	Armenia	–	AF145951	g

Table 1. (Continued)

Species	Fig. 1	River	Locality/Region	ATP synthase6/8	Cytochrome *b*	Source
Capoeta trutta	17	Tigris	Turkey	–	AF145949	f
Luciobarbus albanicus	24	Trichonis	Panetolio (Greece)	AY004690	AY004723	b
Luciobarbus antinorii	13	Bichri	Fatnassa (Tunisia)	AY004692	AY004725	b
Luciobarbus biscarensis	11	El Abiod	Arris (Algeria)	AY004693	AY004726	b
Luciobarbus biscarensis amguidensis	14	Imirhou	El Tassili, Iherir (Algeria)	AY004691	AY004724	b
Luciobarbus bocagei	42	Duratón	Carrascal del Río (Spain)	AY004695	AY004728	b
Luciobarbus brachycephalus	18	Terek	Kislar (Russia)	AY004696	AY004729	b
Luciobarbus callensis	12	Kebir	Ain Assel (Algeria)	AY004680	AF045974	b,d
Luciobarbus capito	18	Terek	Kislar (Russia)	AY004681	AF045975	b,d
Luciobarbus comizo	43	Tajo	Colmenar de Oreja (Spain)	AY004702	AY004735	b
Luciobarbus esocinus	16	Tigris	Diyarbakir (Turkey)	–	AF145934	f
Luciobarbus graecus	23	Kifisos	Orhomenos (Greece)	AY004684	AF090786	b,d
Luciobarbus graellsii	41	Gállego	Ipiés (Spain)	AY004683	AF045973	b,d
Luciobarbus guiraonis	44	Buyent	Pego (Spain)	AY004685	AF045972	b,e
Luciobarbus ksibi	3	Kasab	Essaouira (Morocco)	AY004705	AY004738	b
Luciobarbus labiosa	6	Ifrane	Azrou (Morocco)	AY004700	AY044733	b
Luciobarbus lepineyi	1	Noun	Iguissel (Morocco)	AY004706	AY004739	b
Luciobarbus longiceps	15	Tiberias Lake	Israel	–	AF145942	f
Luciobarbus magniatlantis	4	Bounual	Bounual (Morocco)	AY004714	AY004747	b
Luciobarbus massaensis	2	Assaka	Assaka (Morocco)	AY004707	AY004740	b
Luciobarbus microcephalus	45	Estena	Navas de Estena (Spain)	AY004686	AF045971	b,e
Luciobarbus moulouyensis	7	Moulouya	Boumia (Morocco)	AY004709	AY004742	b
Luciobarbus mursa	20	Arax	Armenia	–	AF145943	g
Luciobarbus nasus	5	Oum Er Rbia	El Borj (Morocco)	AY004711	AY004744	b
Luciobarbus pallaryi	8	Guir	Boudenib (Morocco)	AY004712	AY004745	b
Luciobarbus mystaceus	21	Keban Dam Lake (Euphrates river)	Elazig (Turkey)	–	AF145938	f
Luciobarbus sclateri	46	Alhama	Granada (Spain)	AY004688	AF045970	b,e
Luciobarbus setivimensis	10	Aissi	Azouz (Algeria)	AF317412	AY015991	b
Luciobarbus sp.4	9	Tifrit	Balloul (Algeria)	AY004710	AY004743	b
Luciobarbus subquincunciatus	21	Euphrates	Elazig (Turkey)	–	AF145937	f
Barbonymus gonionotus	–	Moon	Ubon (Thailand)	AB238966	AB238966	c
‚Barbus' anoplus	–	Incomati	Ngodwana (South Africa)	AF287361	AF287417	a
‚Barbus' bigornei	–	Kaba	Kouloundela (Guinea Conakry)	AY004719	AY004752	b
‚Barbus' bynni bynni	–	Nile	Egypt	AF287366	AF287420	a
‚Barbus' fritschii	–	Zamrine	Khouribga (Morocco)	AF287380	AF287429	a

Table 1. (Continued)

Species	Fig. 1	River	Locality/Region	ATP synthase6/8	Cytochrome *b*	Source
‚Barbus' gurneyi	–	Mgeni	Kwazulu-Natal (South Africa)	AF287383	AF287432	a
‚Barbus' motebensis	–	Marico	North Western Province (South Africa)	AF287387	AF287435	a
‚Barbus' paludinosus	–	Mooi	North Western Province (South Africa)	AF287388	AF287436	a
‚Barbus' serra	–	Upper Olifants	Western Cape (South Africa)	AF287460	AF287446	a
Carasobarbus canis	–	David	Bet Shean (Israel)	AF288484	AF288486	a
Carassius auratus	–	–	Asia	EF483931	EF483931	h
Cyprinion kais	–	Tigris	Diyarbakir (Turkey)	–	AF180860	g
Cyprinion macrostomus	–	Tigris	Diyarbakir (Turkey)	–	AF180826	g
Cyprinus carpio	–	Lake Biwa	Japan	AP009047	AP009047	i
Gymnocypris przewalskii	–	Qinghai Lake	China	AB239595	AB239595	c
Labeo bata	–	–	India	AP011198	AP011198	j
Labeo batesii	–	Loa Loa	Gabon	AB238967	AB238967	c
Labeo senegalensis	–	–	Africa	AB238968	AB238968	c
Neolissochilus hexagonolepis	–	Trishuli	Central Region, Nepal	EF588118	EF588174	k
Procypris rabaudi	–	Yangtze	Mudong (China)	EU082030	EU082030	l
Pseudobarbus afer	–	Blindekloof	Eastern Cape (South Africa)	AF287405	AF287449	a
Pseudobarbus asper	–	Vlei	Western Cape (South Africa)	AF287407	AF287451	a
Pseudobarbus phlegeton	–	Noordhoeks	Western Cape (South Africa)	AF287408	AF287452	a
Puntius conchonius	–	Aquarium	Asia	AY004718	AY004751	b
Puntius ticto	–	Mae Kok	Chang Rai (Thailand)	AB238969	AB238969	c
Puntius titteya	–	Aquarium	Asia	AF287411	AF287455	a
Schizothorax zarudnyi	–	Sistan	Southeastern Iran	EF588136	EF588192	k
Sinocyclocheilus altishoulderus	–	–	China	FJ984568	FJ984568	m
Sinocyclocheilus grahami	–	–	China	GQ148557	GQ148557	m
Tor tambroides	–	Phetchaburi Province	Thailand	EF588111	EF588167	k
Varicorhinus maroccanus	–	Oum Er Rbia	El Borj (Morocco)	AF287413	AF287457	a

Sources are: a: Machordom & Doadrio (2001b), b: Machordom & Doadrio (2001a), c: Saitoh et al. (2006), d: Zardoya & Doadrio (1999), e: Zardoya & Doadrio (1998), f: Tsigenopoulos et al. (2003), g: Durand et al. (2002), h: Lee (unpublished), i: Mabuchi et al. (2006), j: Saitoh et al. (2011), k: Nguyen et al. (2008), l: Zhang et al. (2009), m: Wu et al. (2010).

Table 1. List of species included in the present study. Geographical origin is indicated when known and shown in Fig. 1 for species of *Barbus*, *Luciobarbus*, *Capoeta* and *Aulopyge*. Color codes refer to geographical range of the species irrespective of generic placement: Northern Africa, Anatolia, Middle East and Caucasus, Greece, central and eastern Europe, and Balkans, Italy, Iberia, Asia, and central and southern Africa. Accession numbers of the two mitochondrial genes analyzed are shown.

4. Phylogeny of barbels

4.1 Relationships among genera and major groups

The phylogenetic relationships of circum-Mediterranean barbels have been thoroughly explored over the last decades. The wide phylogeny of barbels obtained here based on mitochondrial cytochrome *b* and ATPsynthase 6/8 genes, which includes several additional allied Asian and African cyprinines, is shown on Fig. 2. It is consistent with phylogenies obtained in previous studies based on partially overlapping sets of taxa (Durand et al., 2002; Gante et al., 2009; Machordom & Doadrio, 2001a, 2001b; Mesquita et al., 2007; Tsigenopoulos & Berrebi, 2000; Tsigenopoulos et al., 2002, 2003, 2010; Zardoya & Doadrio, 1998, 1999).

Circum-Mediterranean *Barbus s. str.* forms a strongly supported monophyletic group composed of two barbel lineages (Machordom & Doadrio, 2001a). These mitochondrial lineages, *Barbus* and *Luciobarbus*, now considered distinct genera, are in agreement with previous morphological evidence (Doadrio, 1990), and recent nuclear data (Gante, 2009). *Barbus* and *Luciobarbus* are sister to *Aulopyge* as initially suggested by Howes (1987) and Tsigenopoulos & Berrebi (2000). Altogether, they are likely sister to a group constituted by Middle Eastern *Cyprinion*, and Asian genera such as *Schizothorax* (Durand et al., 2002) and *Gymnocypris* (Fig. 2). This relationship is not well supported by available molecular evidence and would benefit form added sampling effort, both in terms of taxa and markers.

Mitochondrial phylogenies strongly indicate that 'true' barbels are not closely related to other African and Asian barbels. Rather, African diploids are a distinct paraphyletic group, with African tetraploids nested within them, suggesting a tetraploidization event from African diploids, and independent from the one that originated circum-Mediterranean barbels (Fig. 2; Machordom & Doadrio, 2001a; Tsigenopoulos et al., 2002). Likewise, hexaploid cyprinins found in Africa and the Middle East constitute an independent evolutionary lineage whose origin is still not well understood (Fig. 2). These have recently been lumped into *Labeobarbus* (Tsigenopoulos et al., 2010). Together with *Labeo*, they indicate multiple independent colonization events of Africa from Asian ancestors, possibly at different times.

4.2 Phylogenetic diversity of circum-Mediterranean barbels

Even without exhaustive sampling, the genera *Aulopyge*, *Barbus* and *Luciobarbus* show strikingly different degrees of diversity. *Aulopyge* is a species-poor genus composed of only one very specialized extant species, *A. huegelii*, compared to the species-rich *Barbus* and *Luciobarbus*. *Aulopyge heugelii* is an (almost) scale-less species with an elongated urogenital opening which functions as an ovipositor (Kottelat & Freyhof, 2007). It inhabits the Dinaric karst in the Balkanic region. Perhaps the lack of diversity within the genus can be explained by its biology and the degree of specialization attained and/or by its habitat, being imprisoned within the karstic labyrinth and not able to colonize other regions.

The genus *Barbus* is composed of at least four mitochondrial lineages (Fig. 2). It is restricted to the north Mediterranean region and is particularly diverse in Greece where species belonging to three out of the four identified lineages are found. Low diversity of *Barbus* in central and eastern Europe is likely related to the last Ice Ages, when glacier formation drove local populations/species to extinction, followed by rapid re-colonization by a restricted pool of founders (Kotlík & Berrebi, 2001).

The genus *Luciobarbus* is the most diverse and widespread of all. It is composed of at least seven mitochondrial lineages showing very good geographic concordance (Fig. 2). This pattern indicates that *Luciobarbus* speciated *in loco* after seeding by ancestral species.

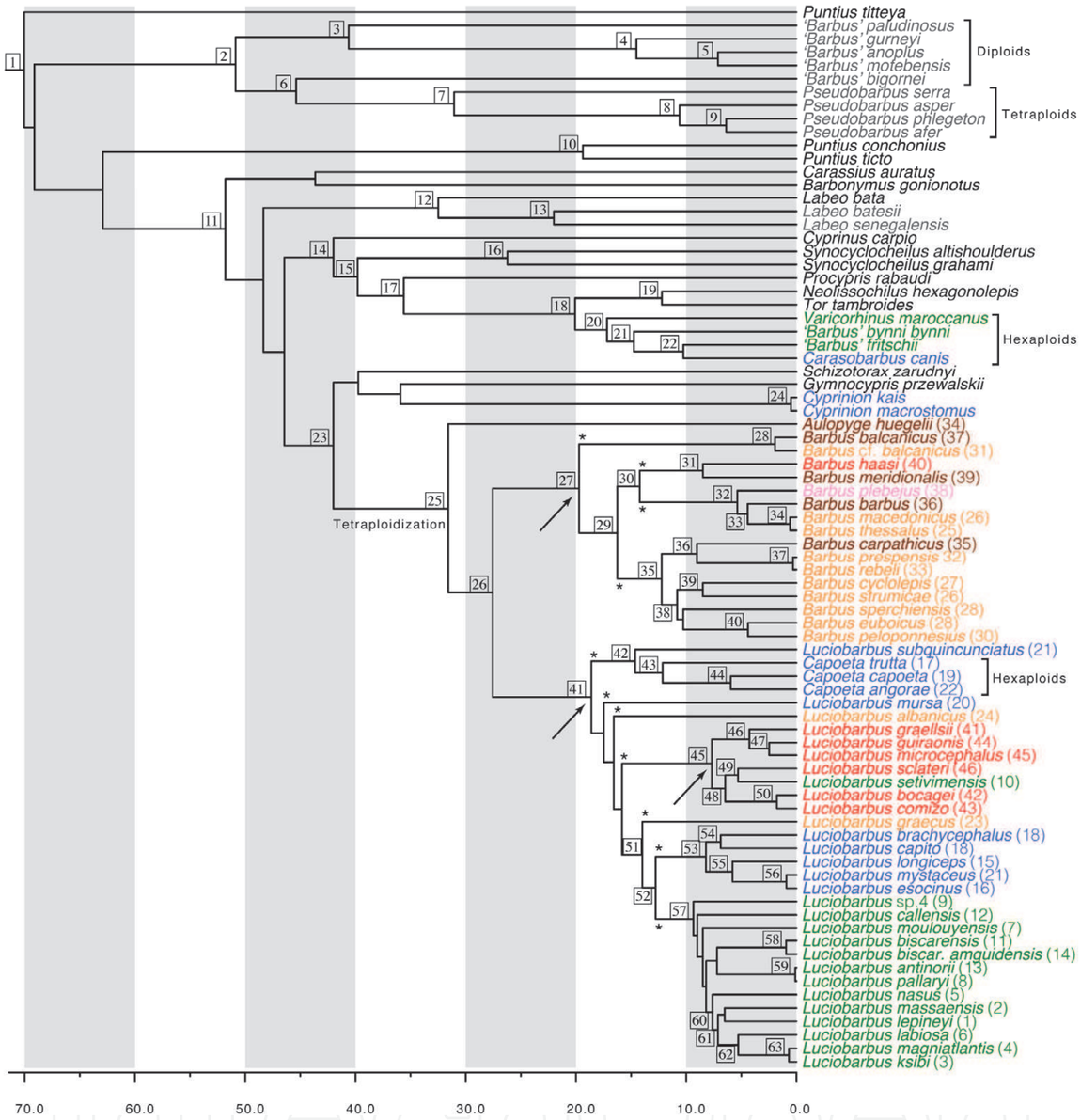

Fig. 2. Bayesian phylogeny calibrated using fossils of *Barbus*, *Luciobarbus* and Iberian *Luciobarbus* (arrows). Nodes with posterior probabilities above 0.8 are numbered in boxes and shown in Table 2. Asterisks (*) label lineages identified within *Barbus* and *Luciobarbus*. Color codes refer to geographical range of the species irrespective of generic placement: Northern Africa, Anatolia, Middle East and Caucasus, Greece, central and eastern Europe, and Balkans, Italy, and Iberia, Asia, and central and southern Africa. Numbers after species names refer to Fig.1 and Table 1.

Interestingly, one of these lineages comprises the hexaploid genus *Capoeta*. *Capoeta* appears to be monophyletic and has likely evolved from ancestors of (the tetraploid?) *Luciobarbus subquincunciatus*. Together, they form a strongly supported monophyletic group deep in the *Luciobarbus* lineage (Durand et al., 2002; Tsigenopoulos et al., 2003). *Luciobarbus* has a very wide distribution, only not being native to central and eastern Europe, and Italy (Doadrio,

Node No.	Age (Ma)	95% HPD	Posterior Probability
1	70.0	57.0–89.9	1
2	50.9	41.0-62.2	1
3	40.6	31.5-51.2	1
4	14.5	10.4-18.8	1
5	7.1	4.7-10.1	1
6	45.4	35.5-56.6	0.88
7	31.1	23.0-40.4	1
8	10.6	7.4-14.3	1
9	6.4	4.0-9.1	1
10	19.4	12.6-27.3	1
11	51.8	44.2-60.3	1
12	32.5	25.5-40.4	1
13	22.0	16.3-29.3	1
14	42.0	35.2-48.5	1
15	39.8	33.3-46.3	0.86
16	26.2	20.0-33.0	1
17	35.6	29.4-41.8	1
18	20.1	15.9-24.5	1
19	12.2	8.0-17.1	1
20	17.2	13.4-21.2	1
21	14.8	11.3-18.6	0.91
22	10.3	7.1-13.7	1
23	42.0	36.2-49.1	0.86
24	0.5	0.1-1.2	1
25	31.6	27.4-36.2	1
26	27.6	24.6-31.2	1
27	19.7	19.2-20.5	1
28	1.9	1.1-3.2	1
29	16.3	14.0-18.4	0.98
30	14.2	11.4-16.8	0.80
31	8.5	5.6-11.6	1
32	5.4	3.6-7.3	1
33	4.4	2.9-6.2	0.95
34	0.6	0.2-1.1	1
35	12.2	10.1-14.5	1
36	9.0	6.6-11.5	1
37	0.3	0.1-0.7	1
38	10.8	8.7-12.9	1
39	8.5	6.2-10.7	1
40	4.4	2.7-6.3	1
41	18.6	17.4-19.7	1
42	14.6	10.9-18.0	0.99
43	12.1	8.6-16.0	0.99
44	6.0	3.3-9.0	1
45	7.7	6.4-9.4	1
46	4.3	2.9-5.8	1

Table 2. (Continued)

Node No.	Age (Ma)	95% HPD	Posterior Probability
46	4.3	2.9-5.8	1
47	2.5	1.5-3.7	1
48	6.5	5.0-8.1	0.84
49	5.3	3.7-7.1	1
50	1.8	1.0-2.9	1
51	14.0	11.5-16.3	1
52	12.8	10.7-15.1	0.97
53	8.2	5.7-10.6	1
54	6.9	4.6-9.3	0.94
55	5.8	3.6-8.4	1
56	0.9	0.3-1.8	1
57	9.3	7.6-11.2	1
58	0.9	0.4-1.6	1
59	0.1	0.0-0.3	1
60	8.2	6.7-9.7	0.99
61	7.6	5.6-8.6	0.85
62	7.1	3.8-6.8	1
63	0.7	0.3-1.2	1

Table 2. Ages and their 95% HPD of tree nodes (Fig. 2) with posterior probability above 0.8.

1990). It is very diverse in northern Africa, where one lineage radiated and where a colonizer from Iberia, *L. setivimensis*, can be found (Machordom & Doadrio, 2001b). *Luciobarbus* is also relatively diverse in the Middle East and Caucasus, where at least three lineages occur, concordant with multiple colonization routes scenario (Almaça, 1990). *Capoeta* is also a very species-rich genus, with about 20 species distributed from western Asia to Anatolia (Banarescu, 1999; Turan et al., 2008).

Besides the inferred radiation within Africa, *Luciobarbus* has undergone rapid speciation early in its existence – most of the lineages identified date back to early *Luciobarbus* diversification (Tsigenopoulos et al., 2003). These polytomies (as the ones identified in the African lineage) do not likely represent a lack of information content in the data (soft polytomies), since the phylogenetic signal before and after these splits is very strong (Fig. 2). Therefore, these polytomies should represent legitimate radiation events (i.e. hard polytomies).

Regarding regional relationships within *Luciobarbus*, the northern African lineage, the Middle Eastern/Caucasus lineage and the Greek *L. graecus* form a strongly supported group. This is in conflict with the view that Iberia could have been seeded by northern African *Luciobarbus* (Doadrio, 1990; Gante et al., 2009) or the other way around (Almaça, 1990). Likewise, a hypothetical relationship between *Capoeta* and Iberian *Luciobarbus* (Tsigenopoulos et al., 2003) is not supported by the data. This lends the exact origin and relationships of Iberian *Luciobarbus* a mystery.

In contrast to this abundance of fast speciation in *Luciobarbus*, only a few short internodes are present in *Barbus* lineage. Whether this pattern reflects a difference in biology between *Barbus* and *Luciobarbus* is unclear. Interestingly, though, poorly supported nodes show some overlap in time, suggesting a possible external (environmental) driver. This hypothesis would need proper testing with a much more exhaustive taxon sampling.

Regarding regional relationships within *Barbus*, there is a much weaker correlation between lineages and geography than that seen in *Luciobarbus*. Such pattern indicates less isolation between Greece, central and eastern Europe, Italy and Iberia.

5. Dating the diversification of barbels

The dating strategy followed here differs from that of most other studies that included circum-Mediterranean barbels. Here, up to date fossil data (Böhme & Ilg, 2003) was used to calibrate a molecular clock, instead of biogeographical events or 'standard' mutation rates. As a consequence, the dates estimated in the present work are substantially older than previous estimates. For instance, the time of splitting between *Barbus* and *Luciobarbus* has been estimated to have occurred 5.5 Ma (Machordom & Doadrio, 2001b), 7.3 Ma (Tsigenopoulos et al., 2010) or ≈8 Ma (Tsigenopoulos et al., 2003; Zardoya & Doadrio, 1999) using the Messinian Salinity Crisis as the driver of speciation of the Iberian *Luciobarbus* lineage. It was estimated to have occurred 10.3 Ma (Tsigenopoulos et al., 2010) or 10.6–12.8 Ma (Tsigenopoulos & Berrebi, 2000) using previous estimates of mutation rates. Since the oldest known fossils of *Barbus* and *Luciobarbus* are 19.0 Ma and 17.7 Ma, respectively, those ages are certainly an underestimation of the real time of divergence between these two genera. In contrast to the abovementioned estimates, according to the calibration used here, *Barbus* and *Luciobarbus* diverged 27.6 Ma (95% HPD: 24.6–31.2 Ma). Since the calibration was applied to the nodes (without the stem), it is possible this age could be somewhat overestimated if earlier lineages diversifying within both *Barbus* and *Luciobarbus* (and represented in the fossils found) got extinct and are missing from the molecular phylogeny. Nevertheless, other sources of evidence support the new estimates shown here. For instance, divergence of *Varicorhinus* is estimated to have occurred 17.2 Ma (95% HPD: 13.4–21.2 Ma), which is supported by fossils of 17.8 Ma found in central Europe (Böhme & Ilg, 2003). Furthermore, the estimated time of divergence of *L. setivimensis* is 5.3 Ma (95% HPD: 3.7–7.1 Ma), which is exactly coincident with the re-opening of the Strait of Gibraltar by the end of the Messinian (Krijgsman et al., 1999).

According to the molecular clock calibration presented here, divergence of the lineage leading to *Aulopyge* happened 31.6 Ma (early Oligocene, Rupelian) and divergence between *Barbus* and *Luciobarbus* occurred 27.6 Ma (late Oligocene, Chattian). After a long period of stasis (or possibly high extinction) diversification within *Barbus* and *Luciobarbus* took place 19.7 Ma and 18.6 Ma, respectively (early Miocene, Burdigalian). The lineage leading to *B. haasi* and *B. meridionalis* split 14.2 Ma (middle Miocene, Langhian–Serravalian boundary). Nevertheless, this date should be carefully interpreted since the node support is rather low (BI = 0.80). Indeed, fossil teeth and vertebrae of *Barbus* have been found in Iberian sediments with 16–17 Ma (Doadrio 1990). *Barbus haasi* and *B. meridionalis* subsequently diverged at 8.5 Ma (late Miocene, Tortonian). The lineage leading to the Italian *B. plebejus* (and *B. tyberinus*; Tsigenopoulos & Berrebi, 2000) split 5.4 Ma (late Miocene, Messinian). *Capoeta* split from *L. subquincunciatus* 14.6 Ma (middle Miocene, Langhian) and started diversifying 12.1 Ma (middle Miocene, Serravalian). Between 18.6 Ma and 14.0 Ma (Miocene, Burdigalian–Langhian boundary) a series of fast cladogenetic events took place within *Luciobarbus*. One of them involved the ancestor of Iberian taxa, which only started diversifying 7.7 Ma (late Miocene, Tortonian–Messinian boundary). Again, like in the case of the apparent evolutionary stasis experienced by *Barbus* and *Luciobarbus* immediately after their split, it is possible that such long branch represents high extinction rather than low speciation rate. This time of divergence of Iberian *Luciobarbus* is coincident with the split of the Iberian *B. haasi*. As mentioned above, *L. setivimensis* split from its Iberian sister 5.3 Ma (Miocene-Pliocene, Messinian–Zanclean boundary). At 12.8 Ma (middle Miocene, Serravalian) the northern African and Middle Eastern *Luciobarbus* split. Rapid radiation within the northern African lineage took place between 9.3 Ma and 8.2 Ma (late Miocene, Tortonian). Therefore, *Aulopyge*, *Barbus* and *Luciobarbus* originated during the Oligocene, all lineages (including

Capoeta) arose in the Miocene and no radiation events date to the Messinian Salinity Crisis of the Mediterranean.

6. Paleogeography of the Mediterranean Basin and diversification of barbels

Seeding of the Mediterranean with a tetraploid barbel lineage most likely occurred during the late Eocene or early Oligocene (Fig. 2). This dating is consistent with an Asian origin of cyprinids and colonization of Europe at the closing of the Turgai Strait in the Eocene–Oligocene boundary (Almaça, 1990; Banarescu, 1992; Briggs, 1995; Rögl, 1999). Progression towards the west was possible due to the emergence of a large landmass that extended across the Balkans, Anatolia and Iran (Rögl, 1999). The carbonate rocks with more than 8,000 m that form the Karst Dinarides were deposited for more than 270 Ma and raised during the Alpine orogeny (Velic, 2007), suggesting that present-day karst habitat inhabited by *Aulopyge* was already present, in the place this oldest barbel lineage is presently found. Such a colonization scenario through southwestern Asia was also hypothesized for Leuciscins (Perea et al., 2010).

The split between *Barbus* and *Luciobarbus* in the late Oligocene could have been driven by the fragmentation of this landmass (Rögl, 1999). In a time of intense tectonic activity in the Mediterranean, the opening of the Slovenian corridor is a likely candidate, fragmenting *Barbus* to the north and *Luciobarbus* to the south. The timing of diversification within these genera at 20 Ma is coincident with the closure of the Slovenian seaway (Rögl, 1999). This reunited landmass might have allowed access to regions where the oldest fossils of *Barbus* and *Luciobarbus* have been found.

The time of origin of the lineage leading to *L. subquincunciatus* and *Capoeta*, which are found in the Middle East and Caucasus, is coincident again with a transitory fragmentation of this landmass. In the middle Miocene, seaway corridors opened between Arabia, south Anatolia and eastern Anatolia, and possibly along a suture between the Balkanides and the Rhodopes (Rögl, 1999). The branching out of *Capoeta* occurred at the time when oceanic circulation between the Indian and Atlantic oceans stopped, in the Serravalian (Rögl, 1999).

By then, the main lineages within *Barbus* and *Luciobarbus* had already originated. Explaining their current distribution is no easy task with current paleogeographical and paleontological evidence. Fossils of *Barbus* of Burdigalian age are found from Turkey to Iberia (Böhme & Ilg, 2003; Doadrio, 1990), which corresponds to the present distribution of the genus, except for Italy. Colonization of Italy during the Messinian Salinity Crisis is a likely scenario. Equally old fossils of *Luciobarbus* have been only found in Turkey and it is not before the Tortonian they are found in central Europe (Böhme & Ilg, 2003). This could be due to taxonomic bias, since *Luciobarbus* has not been recognized for as long as *Barbus*, or it could reflect a real trend of *Luciobarbus* biogeography. Nevertheless, the presence of fossils in central Europe in Tortonian times, where it is now absent, opens new routes for *Luciobarbus* dispersal. In particular, it is known that during Alpine orogeny, marine influence in the North Alpine Molasse ended in the middle Miocene (Langhian; Hsü et al., 1977; Krenmayr, 1999; Rögl, 1999). Barbels could have used this basin as a means of southwestward dispersion to Iberia, independent from the colonization of northern Africa. Alternatively, they could have used slightly different pathways via the *Gomphotherium* landbridge connecting Africa and Eurasia (Rögl, 1999), as suggested by Perea et al. (2010) to explain vicariance of Peloponessus and Magreb *Tropodophoxinellus*. Since the distribution areas of Leuciscinae and Cyprininae are similar, as well as inferred dates of groups occupying those regions, it is likely they shared common migratory routes. The subsequent radiation of northern African *Luciobarbus* is likely related to complex paleogeomorphology of the Rif massif (e.g. Alvinerie et al., 1992; Machordom &

Doadrio, 2001a). Starting around 7.8 Ma the marine corridors between Iberia and northern Africa became restricted until the establishment of a land bridge around 5.6 Ma (Messinian) (Garcés et al., 1998, 2001; Krijgsman et al., 1999; Martín et al., 2001; van Assen et al., 2006). Dispersal and subsequent vicariance of *L. setivimensis* between Betic and Riffian massifs has occurred during the Messinian Salinity Crisis. Nevertheless, speciation events are not concentrated in this period, nor are the inferred radiations. Other authors have recently ruled-out a "Lago Mare dispersal" for leuciscin cyprinids (Levy et al., 2009; Perea et al., 2010). This period seems to have been used for transfer between adjacent areas (e.g., Iberia – northern Africa, central Europe – Italy) rather than a Mediterranean-wide colonization by barbels.

7. Conclusion

According to the fossil calibrated molecular phylogeny presented here, divergence of the circum-Mediterranean barbel lineages occurred during the Oligocene. Divergence within *Barbus* and *Luciobarbus* took place throughout the Miocene, including spreading to new areas. Altogether, colonization of the Mediterranean region by barbels must have been a very dynamic process we are just starting to understand, as indicated by the presence of many fossils in regions where the genera are presently not found. A good example is the presence of several *Luciobarbus* fossils in Libya, Italy, Austria, and Slovakia.

Greater insight will likely continue coming from paleontological and paleogeographical data, and that should be accompanied by new biological data of extant species. In particular, all of these scenarios are only based in non-recombinant mitochondrial DNA markers. The coming decade should see the rise of nuclear phylogenies and an improved understanding of barbel biogeography in the Mediterranean region.

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As everybody knows, the dynamic interactions between biotic and abiotic factors, as well as the anthropic ones, considerably affect global climate changes and consequently biology, ecology and distribution of life forms of our planet. These important natural events affect all ecosystems, causing important changes on biodiversity. Systematic and phylogenetic studies, biogeographic distribution analysis and evaluations of diversity richness are focal topics of this book written by international experts, some even considering economical effects and future perspectives on the managing and conservation plans.

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